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Application Note

Eshmuno® Fit

Custom Affinity Resin Development Services to Streamline Downstream Processing of Difficult to Purify Molecules

Affinity chromatography is used in downstream purification workflows to separate a molecule of interest from a crude feed stream. A ligand immobilized on the chromatography resin captures the target molecule via a reversible, specific binding interaction and can deliver up to 90% purity in a single step. The downstream purification template for monoclonal antibodies (mAbs) typically includes Protein A affinity chromatography in which the immobilized Protein A ligand captures mAbs via their Fc regions. In cases where the target molecule does not have an Fc region, this templated process cannot be applied, necessitating lengthy process development to create a tailored approach. In many cases, the resulting purification scheme can be complicated, requiring a combination of hydrophobic interaction and ion exchange chromatography, with a high cost of goods (COGs) and low yields due to the cumulative loss of target molecule at each step.

Use of custom affinity resins with novel ligands enhances selectivity for challenging molecules and can thus simplify downstream purification schemes. These resins can be used to capture any stable molecule such as non-traditional mAbs, peptides, enzymes, vaccines, and adeno-associated virus (AAV) vectors used to deliver gene therapies. By incorporating custom affinity capture, the number of downstream steps can be reduced, leading to lower costs and improved yields (Figure 1). This approach also offers the potential to establish a purification template for similar molecules that can be captured using the same custom affinity resin.

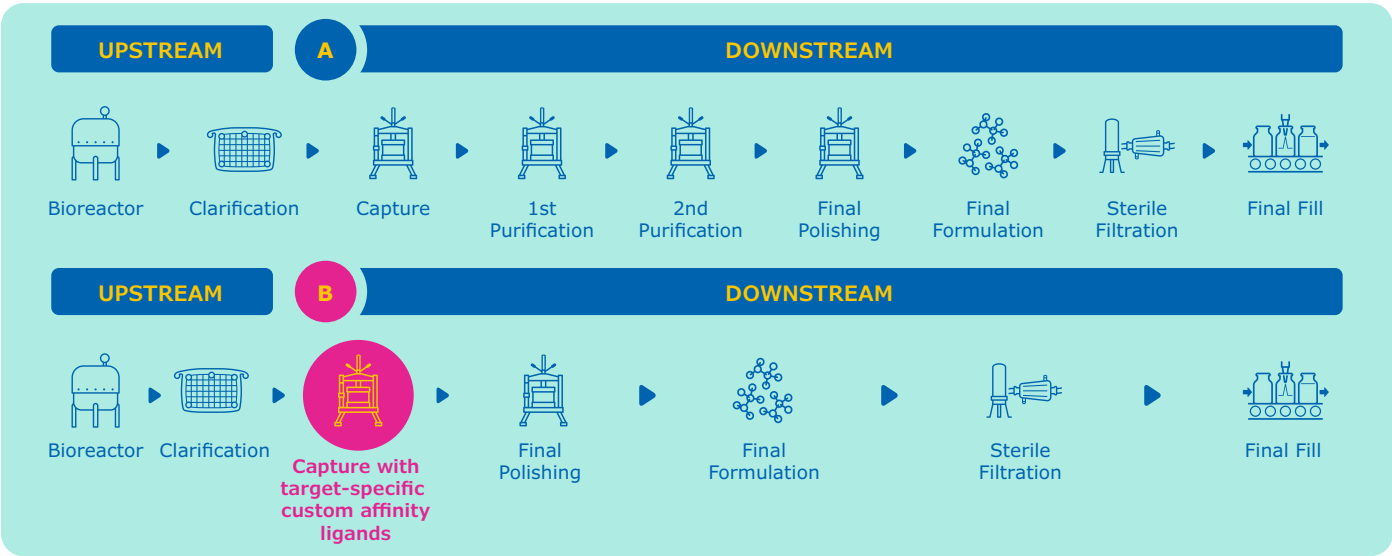

Figure 1.

Non-templated processes (A) lead to long timelines for process development and have complicated purification schemes leading to high costs and low yields. Simplifying downstream purification (B) leads to reduced COGs and improved yields.

Nanofitin® Ligands

Eshmuno® Fit custom affinity resins consist of Nanofitin® affitin ligands immobilized on an Eshmuno® matrix. Affitins are small, single-chain, cysteine-free proteins isolated from the hyperthermophile archaeon *Sulfolobus acidocaldarius*.[1] Randomization of the 14 amino acid DNA-binding site yields large libraries (~1e^{14}) of high specificity ligands that can be isolated in a time-efficient manner[1] (Figure 2). The Nanofitin® library is screened using ribosomal display in which the target molecule is immobilized on magnetic beads and exposed to the library. A selection process based on affinity strength allows implementation of elution conditions that have been previously determined. Screening with ribosomal display offers an important advantage over cell-based approaches as there are no cell-derived impurities that could compromise or delay the screening process. The primary results of the first screening are available within a month.

Screening can be directed against a specific epitope that is fixed across multiple target molecules or against random epitopes if the structure of the target molecule may vary somewhat. In all cases, the ligands are thermostable, reusable, bearing tailored resistance to moderate caustic conditions, and can be readily conjugated to chromatographic resins. Ligands can be manufactured in *E. coli* using a simple and cost-effective process. Following the screening process, the customer receives three prototypes of custom affinity resins to evaluate along with recommended buffer conditions (Figure 3). If some specifications of the generated prototypes cannot be fulfilled, another round of ribosomal display is performed.

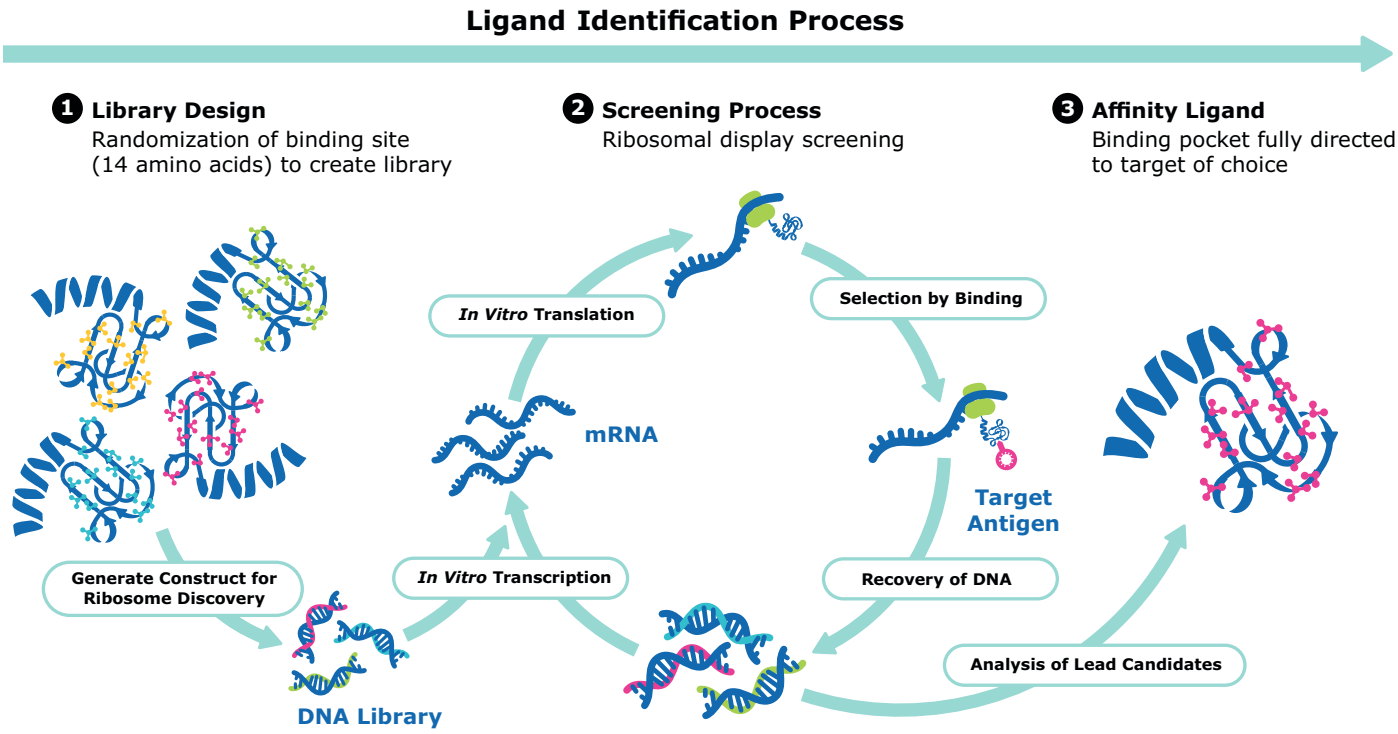

Figure 2.

The Nanofitin® ligand identification and screening process using ribosomal display.

In addition to minimizing downstream processing time and increasing recovery yield, custom affinity resins can be used as a platform approach for a variety of molecules by simply changing the immobilized ligand that confers specificity to the affinity capture step (Figure 3).

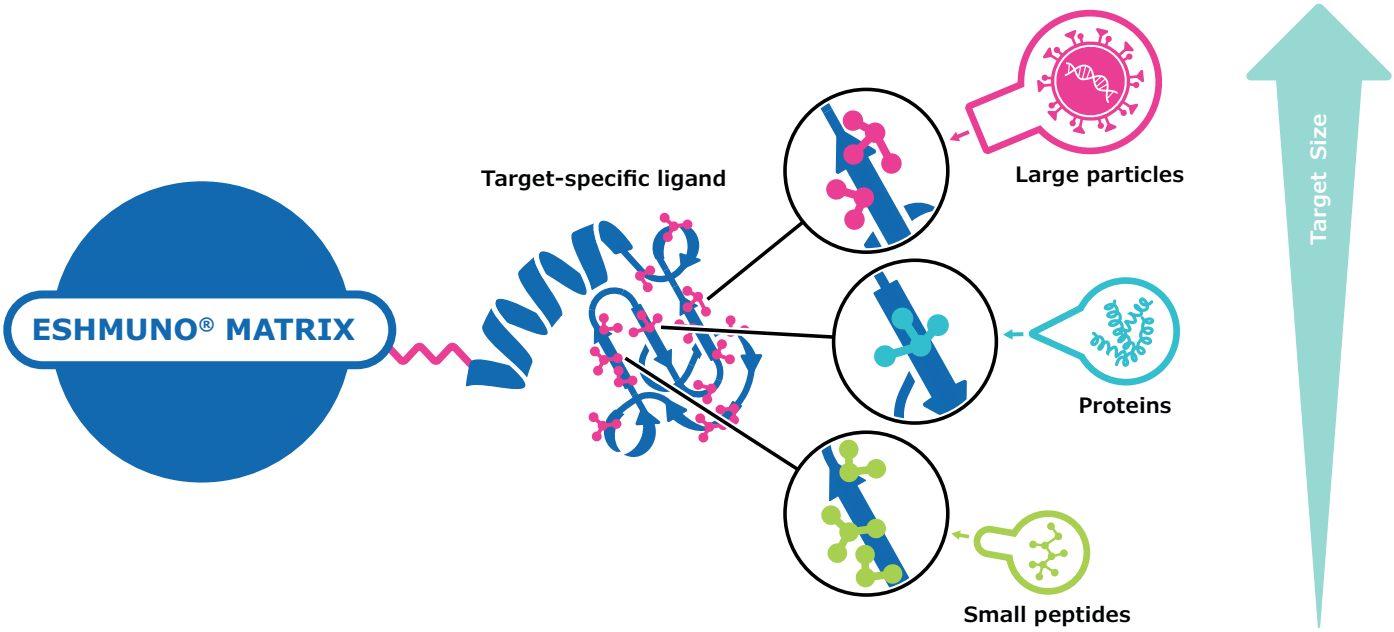

Figure 3.

An efficient process is used to produce the first target specific affinity resin samples.

Case Study: Affinity Capture of a Vaccine Candidate Molecule

The following case study describes the one-step purification of a recombinant, mutant form of streptolysin O (SLO)[3], a vaccine candidate against *Streptococcus pyogenes*; complete experimental details and results were by published by Chevrel, 2022.[2] Purification of this protein requires removal of product-related impurities, host cell DNA, and host cell proteins. The existing purification method required three orthogonal chromatographic steps to achieve the specified purity, each of which resulting in associated product loss.[3]

Nanofitin® Ligands Discovery and Characterization

A Nanofitin® ligand library was challenged through four rounds of ribosome display using the recombinant SLO protein. A total of 180 clones were screened by ELISA and 21 having a favorable signal-to-noise ratio (>1.5) were identified (Figure 4a). Positive clones were sequenced and classified into six different families based on the homology of their binding sites (Figure 4b). The ten ligands with the highest ELISA signals were further characterized for expression yield, binding kinetics and stability when exposed to caustic conditions.

Ligands with a recovery yield of less than 20 mg/L were excluded from characterization (Figure 4c), while the binding characteristics of the remaining ligands were compared using bio-layer interferometry (BLI). All the remaining ligands had affinities ranging from 10 to 100 nM; based on these results, the set of ligand candidates was narrowed to the best clone of each sequence family (NF-TF2-02, -03, -04, and -07). Figure 4d shows the stability of the ligands in accelerated clean-in-place (CIP)-like conditions. Following a 6-hour incubation in NaOH at various concentrations, ligand performance was assessed by ELISA.

Overall, NF-TF2-03 had the best properties in terms of expression yield, affinity, and stability. It was selected as SLO affinity ligand to be incorporated on the chromatographic support.

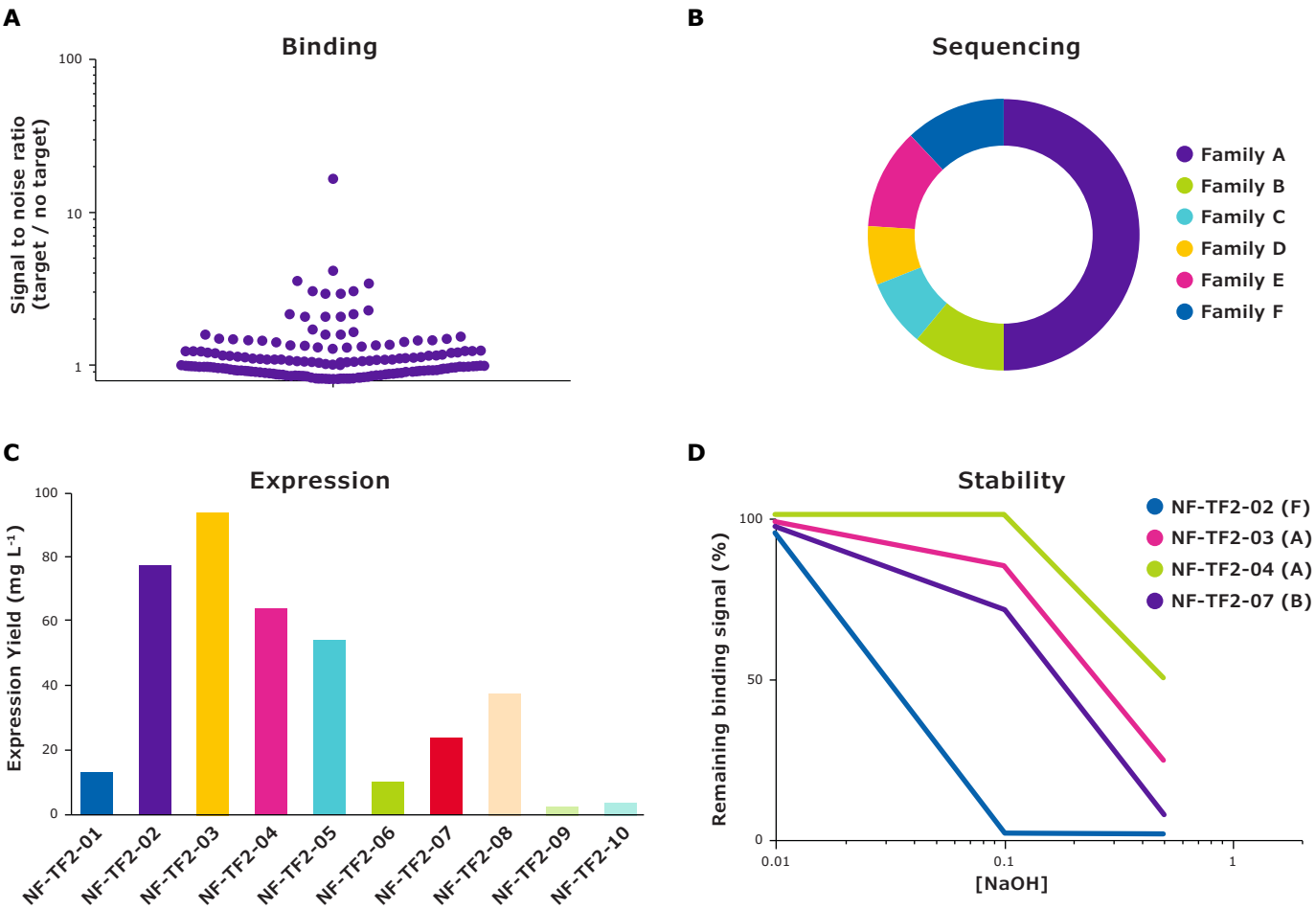

Figure 4.

Enzyme-linked immunosorbent assay (ELISA) screening for streptolysin O (SLO) ligands (A). Distribution of positive Nanofitin® ligands (S/N >1.5) according to their sequences grouped into families based on the homology of their binding site (B). Small-scale expression yield (mg L^{-1}) of the 10 best ligands (C). ELISA-binding signal recovery after accelerated cleaning-in-place (CIP-like) studies (D).

Affinity Process Development

BLI was used to evaluate the dissociation rate (KD) of SLO from NF-TF2-03 in different buffer conditions (Figure 5). NF-TF2-03 was attached to the BLI sensors by its C-terminal cysteine to mimic the orientation on the chromatography resin. Dissociation was complete in about 200 s which predicts an efficient elution once in the column. Optimal binding and elution buffers were set at 10 mM of KH^2PO^4 at pH 7.2, and 0.1 M glycine pH 4.0, respectively.

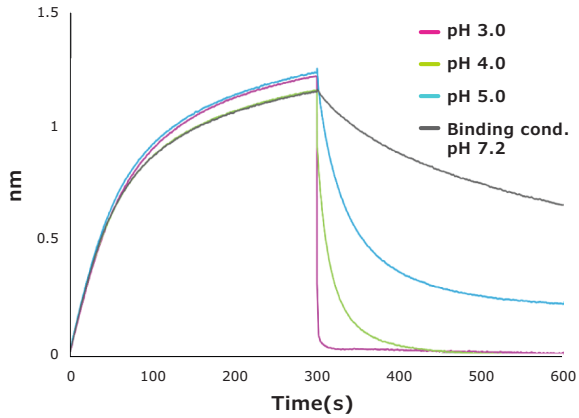

Figure 5.

Dissociation rate (KD) of SLO from NF-TF2-03 in different buffer conditions.

Benefits of One-Step Affinity Purification

The one-step affinity purification process shown in Figure 6 reduced process time and footprint and consistently delivered several important benefits as summarized in Table 1. Product purity was increased (>90%) as well as product yield (0.31 g vs. 0.04 g SLO/kg harvest broth); both values represent a significant improvement over the current three-step process. The DNA/protein ratio was significantly reduced.

The Eshmuno® Fit custom affinity resin column was used over 35 regeneration cycles and maintained capacity above 15 mg/ml. The column and the downstream process have been assessed in a technical run within an industrial setup. Process yield has been improved more than five times with increased purity of the final SLO product.

This custom affinity system can potentially be applied to any biologic for which a specific Nanofitin® is identified, enabling establishment of a platform for more efficient vaccine manufacturing and other molecules.

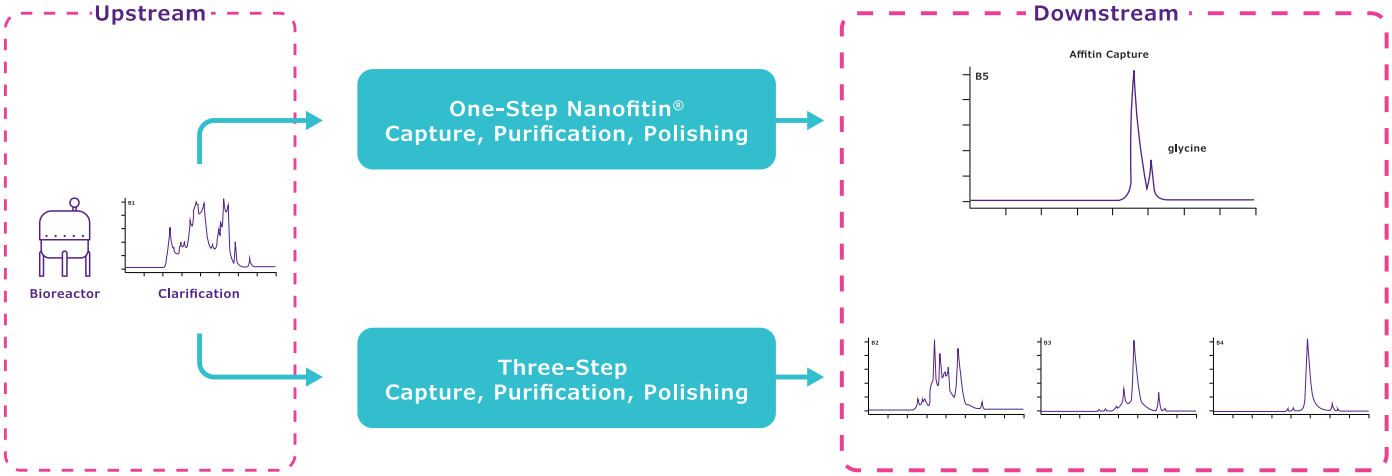

Figure 6.

Comparison of the original three-step recombinant protein purification process and the streamlined, one-step process.

Attributes	Nanofitin® One-Step Capture	Standard 3-Step Process
Purity RP-HPLC (%)	94	90
Integrity SEC (%)	91	84
Purity SDS-PAGE (%)	94	84
HCP western blotting	Negative	Negative
DNA reduction (log)	4.8	2.5
DNA/protein ratio (ppm)	47	29
Bioburden	Negative	Negative

Table 1.

Summary of the benefits provided by the one-step affinity purification process.

Custom Design and Production of Custom Affinity Resins

Nanofitin® ligands offer key advantages for affinity capture due to their high binding capacity and tailored stability in caustic solutions. With a large and highly diverse library of possible affitins and a rapid screening process, development of a customized Eshmuno® Fit resin is an efficient process. Integration of custom Nanofitin® ligands immobilized on an Eshmuno® resin enable the affinity capture of molecules that would otherwise require several purification steps. Downstream processing can be streamlined while achieving similar or better purity as demonstrated in the case study.

The custom affinity resins are available via our project execution framework, outlined in Table 2. It begins with ligand discovery and characterization followed by small-scale functionalization of the Eshmuno® resin. At this point, the customer receives different prototypes and recommended binding and elution buffers. Following customer screening of the samples, a 1 L research sample is produced to support pilot scale development, followed by production of sequentially larger batches, up to 40 L manufacturing batches which are accompanied by regulatory support files. The custom resins are produced in our own facilities, ensuring a robust and resilient supply chain. All steps are coordinated by our dedicated project execution team, ensuring the implementation of the custom affinity resin from start to finish.

Ligand discovery, characterization, and support selection	• Broad ligand screening • Identification of up to 20 ligands • Affinity constant estimation
Small scale immobilization	• Physical characterization • Functional tests • Ability to reuse
1 L research sample generation	• Material for clinical phase I • Specification definition (limited) • Test and release against specification
Synthesis upscaling (3–5 L)	• Specification definition (extended) • Analytical method validation • Ligand scale-up • Process scale-up
Manufacturing under controlled conditions (~40 L)	• Process scale-up to full size • Process validation with three PQ batches • Release of fully commercial material by QA
Regular supply	

Table 2.

The custom affinity resin development process from ligand discovery to commercial supply.

References

1. Mouratou B, et al. (2007). Remodeling a DNA-binding protein as a specific *in vivo* inhibitor of bacterial secretin PulD. Proceedings of the National Academy of Sciences of the United States of America, 104, 17983–17988. https://doi.org/10.1073/pnas.0702963104
2. Chevrel A, et al. (2022). Development of versatile affinity-based system for one step purification process: Case of Group A Strept ococcus vaccine. Biotechnology Bioengineering, 119:3210-3220. DOI: 10.1002/bit.28199
3. Chiarot E, et al. (2013). Targeted amino acid substitutions impair streptolysin O toxicity and group a Streptococcus virulence. MBio, 4, 00387–12. https://doi.org/ 10.1128/mBio.00387-12

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